Biochemical characterization and ligand-binding properties of trehalose-6-phosphate phosphatase from *Mycobacterium tuberculosis*

Lina Shi1, Haiping Zhang2, Yu Qiu1, Qian Wang1, Xueji Wu1, Honghai Wang3, Xuelian Zhang3*, and Donghai Lin1*

1Key Laboratory for Chemical Biology of Fujian Province, College of Chemistry and Chemical Engineering, Xiamen University, Xiamen 361005, China
2Key Laboratory for Cell Biology and Tumor Cell Engineering, College of Life Sciences, Xiamen University, Xiamen 361005, China
3State Key Laboratory of Genetic Engineering, Institute of Genetics, School of Life Sciences, Fudan University, Shanghai 200433, China

*Correspondence address. Tel/Fax: +86-592-2186078; E-mail: dhlin@xmu.edu.cn (D.L.); Tel/Fax: +86-21-55665073; E-mail: xuelianzhang@fudan.edu.cn (X.Z.).

**Trehalose-6-phosphate phosphatase** (TPP) is an essential enzyme for growth of mycobacteria, which has been identified to be a potential anti-tuberculosis drug target. However, the biochemical and ligand-binding properties and the 3D structure of TPP remain unclear so far. In the present study, we expressed the recombinant TPP protein from *Mycobacterium tuberculosis* (*otsB2/Rv3372*). Results from the far-ultraviolet circular dichroism experiments indicated that the secondary structure of TPP was rich in α-helix with a lower structural stability ($C_m = 2.099 \pm 0.134$ M). Ligand-binding assay by isothermal titration calorimetry demonstrated that the recombinant TPP protein could bind with trehalose-6-P in the presence of Mg$^{2+}$ ($K_\text{d} = 39.52 \pm 1.78$ μM) with a molar ratio of 1 : 1. In addition, the 3D structure of TPP was modeled by I-TASSER, indicating that the TPP protein was composed of a hydrolase domain, a cap domain, and an N-terminal domain. Flexible docking was further conducted by using the Simulations/Dock module of the Molecular Operating Environment software. The binding pocket of TPP for both trehalose-6-P and Mg$^{2+}$ was determined, which was located on the interface between the hydrolase domain and the cap domain. Asp149, Gly186, Arg187, Arg291, and Glu295 were identified to be the key residues for TPP binding with trehalose-6-P. This work may lay the basis for further structural and functional studies of TPP and TPP-related novel drug development.

**Keywords**  
Trehalose-6-phosphate phosphatase; trehalose-6-P; circular dichroism; isothermal titration calorimetry; structure model

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**Introduction**

Tuberculosis is usually caused by mycobacteria, which remains a leading infectious disease in the world, with ~8 million new infections and 2.5–3 million deaths per year [1]. As both the control and management of tuberculosis have been complicated by multidrug resistance and latent infection, scientists have been devoting to exploit new immunodominant antigens.

In mycobacteria, trehalose is an integral component of a number of cell wall glycolipids, that can protect proteins and cellular membranes from inactivation or denaturation caused by desiccation, heat, cold, oxidation, and so on [2–5]. As trehalose appears to be essential for virulence of mycobacteria, the reactions involved in the biosynthesis of trehalose might contain potential target sites against tuberculosis. Trehalose can be produced by at least three pathways [6], in which the OtsAB pathway is the dominant one required both for *Mycobacterium tuberculosis* growth in laboratory culture and for virulence in a mouse model [7]. The OtsAB pathway involves condensation of glucose-6-phosphate with uridine diphosphate-glucose to form trehalose-6-phosphate (catalyzed by trehalose-6-phosphate synthase, OtsA), followed by subsequent dephosphorylation generating the free disaccharide [catalyzed by trehalose-6-phosphate phosphatase (TPP), OtsB] [6]. There are two open reading frames, *otsB1* (*Rv2006*) and *otsB2* (*Rv3372*), encoding putative TPP. Previous studies have demonstrated that the *otsB1*-encoding TPP protein does not exhibit detectable phosphatase activity, while the *otsB2*-encoding TPP protein is a functional TPP that is strictly essential for the growth of mycobacteria and provides a tractable target for high throughput screening [7–9]. Furthermore, TPP was reported to be capable of inducing humoral and cellular immune responses [10]. Consequently, TPP has been thought to be a candidate for vaccine development for control of tuberculosis.

There are few studies on the biochemical properties of TPP. It has been demonstrated that TPP is specific for trehalose-6-P as the substrate, and Mg$^{2+}$ is crucial for TPP binding with trehalose-6-P [8]. However, the detailed biochemical and ligand-binding properties as well as the tertiary
structure of TPP remain to be determined. In the present study, we successfully expressed and purified the TPP protein (otsB2/Rv3372) from *M. tuberculosis*. We also addressed the influence of Mg$^{2+}$ and trehalose-6-P on the secondary structure of TPP by far-ultraviolet (UV) circular dichroism (CD) spectroscopy, and measured the binding affinity of TPP for trehalose-6-P by isothermal titration calorimetry (ITC) assay. In addition, we modeled the 3D structure of TPP by the I-TASSER program [11–13], and conducted flexible docking to construct the structural model of the TPP–trehalose-6-P complex.

**Materials and Methods**

**Protein expression and purification**

The expression vector harboring the TPP gene, pET28a-TPP, was transformed into *Escherichia coli* BL21 (DE3). Expression of the target protein was induced in Luria–Bertani media containing 100 μg/ml kanamycin, with 0.2 mM isopropyl-β-D-thiogalactoside for 8 h at 22°C/200 g in cultures until optical density at 600 nm reached 0.6–0.8. The cells were harvested by centrifugation (3500 g for 4 min at 4°C), resuspended in buffer A (50 mM NaH$_2$PO$_4$, 300 mM NaCl, and 10 mM imidazole, pH 8.0), lysed by ultrasonic breaking (400 W for 30 min), and centrifuged (6000 g for 50 min at 4°C). Then, the supernatant was loaded onto an Ni-NTA affinity column (Qiagen, Hilden, Germany) pre-equilibrated with buffer A. After washing the captured column sequentially with 0, 30, and 50 mM imidazole, the His-tag TPP protein was eluted with 250 mM imidazole. The fraction containing the target protein was further purified and desalted by S200 gel filtration (fast protein liquid chromatography) (GE Healthcare, Wisconsin, USA) with buffer B (10 mM Tris-HCl, pH 7.5). The purified protein was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (15%) followed by Coomassie staining.

**Mass spectrometry**

The molecular weight of the recombinant TPP protein was measured on a Bruker microFlex MALDI-TOF-MS spectrometer (Bruker Corporation, Bremen, Germany). The measurement was performed in a positive linear mode with an accelerating voltage of 2000 V. The data were recorded and processed by using the software provided by the instrument.

**CD spectra**

CD experiments were carried out on JASCO J-810 spectropolarimeter (Jasco, Tokyo, Japan) with a quartz cell of 0.1 cm path length at 25°C. For CD experiments, the TPP protein samples (0.2 mg/ml in 60 mM phosphate buffer, pH 7.5) were used. Before measurements, TPP was incubated with 2 mM Mg$^{2+}$ or trehalose-6-P at 25°C for 12 h. The far-UV CD spectra were recorded over the range of 190–250 nm. Each spectrum was the accumulation of three scans with a bandwidth of 1 nm and a step resolution of 0.1 nm at a scan speed of 100 nm/min. All the spectra were processed and corrected for blank.

**Urea-induced unfolding**

TPP proteins were incubated with urea at varying concentrations from 0 to 8.0 M at 25°C for 12 h, then the far-UV CD spectra were recorded, and each spectrum was the average of three consecutive scans. The mean residue molar ellipticities at 222 nm were plotted as the fraction of the unfolded protein. The background corrections were carried out in all spectra by subtracting appropriate blanks.

**ITC measurements**

ITC experiments were performed at 25°C on an isothermal titration calorimeter (ITC200; MicroCal, Piscataway, USA). The energetics of the binding of trehalose-6-P (Sigma-Aldrich, St Louis, USA) to TPP were determined in buffer B with and without Mg$^{2+}$ (2 mM). The reference cell was filled with the corresponding degassed buffer. The TPP protein solution (100 μM) was kept in the sample cell, whereas the working solution of trehalose-6-P (2 mM) was filled in the syringe. A background titration was performed using the identical titrant with buffer B placed in the sample cell. Before loading, all the solutions were degassed. Each experiment was conducted with 17 injections of the working solution (totally 40 μl; 2 μl for the first injection only). Injections were started after baseline stability had been achieved. To ensure a homogeneous mixing in the cell, the stirrer speed was kept constant at 300 rpm. The heat effect per injection was determined by automatic peak integration of the thermal power vs. time curve. The resulting data were fitted by using the Origin software from MicroCal. To account for the heat of dilution, the result of background titration was subtracted from each working solution titration.

**Structure modeling and binding site prediction**

In order to access the tertiary structure of *M. tuberculosis* TPP (otsB2/Rv3372), the structural model of TPP was constructed by using the I-TASSER online server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) [11–13]. Five decoys were generated. The one with the best confidence score (C-score) was selected as the best optimized 3D modeling structure of TPP.

The active binding site was identified by the binding site prediction module of the I-TASSER software, and then flexible docking was conducted by using the Simulations/Dock module of the Molecular Operating Environment (MOE) software (Chemical Computing Group Inc., Canada). The 3D structure of trehalose-6-P (CID: 122336) was downloaded from PUBCHEM and further refined by energy minimization without changing chirality. The following docking
parameters were used: Receptor: Receptor + Solvent, Rescoring1: London dG, Retain: 30, and Refinement: Forcefield (Amber99). Solvation effects were calculated using the reaction field functional form for the electrostatic energy term and a dielectric constant of 4. Ligand–target interaction was calculated by Compute/Ligand interaction Model of MOE.

Results

Sample preparation

In *E. coli* system, the recombinant TPP protein was produced in soluble state. The N-terminal His-tag allowed purification using chromatography on an Ni-NTA column. The fraction eluted by 250 mM imidazole was obtained with a purity of ~80% [Fig. 1(A), lane 7]. After further purification by S200 gel filtration, the homogeneity of the target protein was >95% as judged by SDS–PAGE [Fig. 1(B)]. The yield of the purified TPP protein was about 30 mg per liter of bacterial culture. In addition, analysis of the size-exclusion chromatography indicated that most of the target proteins existed in a monomeric state (eluted at 158.20 min), but a few in a multimeric state (eluted at 118.74 min) [Fig. 1(C)].

Characterization by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry

Mass spectrometry (MS) was used to further identify the TPP protein and check protein purity. The matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS) spectrum indicated that the purity of

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![Figure 1](https://example.com/image1.png)

**Figure 1 Preparation and characterization of TPP**  (A) SDS–PAGE analysis of the purification step of TPP by Ni-NTA column. Lane 1, marker; lane 2, supernatant before loading on Ni-NTA column; lane 3, flow-through after loading; lane 4, fraction washed with 0 mM imidazole; lane 5, fraction washed with 30 mM imidazole; lane 6, fraction washed with 50 mM imidazole; and lane 7, fraction eluted by 250 mM imidazole. (B) SDS–PAGE of purified TPP after size-exclusion chromatography. (C) Size-exclusion chromatography of TPP. The elution was monitored by absorbance at 280 nm. (D) MALDI-TOF mass spectrum of TPP.
The TPP protein was high. The molecular weight of the recombinant TPP protein was measured to be 46,228 Da [Fig. 1(D)]. This value was in accordance with the theoretical molecular weight of 46,236 Da, which was calculated from the amino acid sequence, including residues at the C-terminus (KL) and N-terminus (MGSSHHHHHHSS GLVPRGSHMASMTGGQQMGRGSEFIELV) for cloning and purification purposes.

Secondary structure analysis
The CD spectrum in the wavelength range between 190 and 250 nm is mainly determined by backbone conformation of the protein [14,15]. Therefore, far-UV CD spectroscopy was used to characterize the secondary structure of the recombinant TPP protein. As shown in Fig. 2, the occurrence of two negative peaks around 208 and 222 nm was indicative of the typical secondary structure of a predominantly α-helix protein [16,17]. The secondary structure prediction based on the CD spectrum indicated that the TPP protein was composed of 54.92% α-helix, 8.61% β-sheet, and 36.47% coil (Table 1).

Table 1 Secondary structure elements of TPP predicted from the far-UV CD spectra

<table>
<thead>
<tr>
<th></th>
<th>α-Helix (%)</th>
<th>β-Sheet (%)</th>
<th>Coil (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPP</td>
<td>54.92</td>
<td>8.61</td>
<td>36.47</td>
</tr>
<tr>
<td>TPP + Mg²⁺</td>
<td>59.61</td>
<td>7.68</td>
<td>32.71</td>
</tr>
<tr>
<td>TPP + trehalose-6-P</td>
<td>54.91</td>
<td>8.73</td>
<td>36.36</td>
</tr>
<tr>
<td>TPP + Mg²⁺ + trehalose-6-P</td>
<td>57.61</td>
<td>7.83</td>
<td>34.56</td>
</tr>
</tbody>
</table>

To evaluate the influence of either Mg²⁺ binding and/or trehalose-6-P binding to TPP on the secondary structure of TPP, we compared the far-UV CD spectrum of free TPP with that incubated with Mg²⁺ and/or trehalose-6-P. Results from the secondary structure prediction (Table 1) showed that adding trehalose-6-P to TPP did not significantly influence the secondary structure of TPP, while adding Mg²⁺ to TPP distinctly changed the secondary structure of TPP, implying that Mg²⁺ could somewhat stabilize the structure of TPP and might be crucial for the structural integrity of TPP.

Conformational stability
To access the conformational stability of TPP, we performed urea-induced unfolding transition experiment using far-UV CD spectroscopy. Data were expressed in terms of fraction unfolded \( F_{un} \). Results from the secondary structure prediction showed that adding trehalose-6-P to TPP did not significantly influence the secondary structure of TPP, while adding Mg²⁺ to TPP distinctly changed the secondary structure of TPP, implying that Mg²⁺ could somewhat stabilize the structure of TPP and might be crucial for the structural integrity of TPP.

Ligand-binding assay
ITC provides a direct method to determine thermodynamic parameters for non-covalent, equilibrium interactions between proteins and ligands [18]. The dissociate constant \( K_d \) can be accurately determined by measurement of heat, which is either generated or absorbed once two components bind with each other. In the absence of Mg²⁺, the small, almost constant heat flows were too weak to be measured, indicative of very weak
affinity for TPP binding with trehalose-6-P [Fig. 4(A)]

Contrarily, in the presence of Mg$^{2+}$, the interaction between TPP and trehalose-6-P was more significant [Fig. 4(B)], implying that Mg$^{2+}$ might be crucial for TPP binding with trehalose-6-P. Each peak represents a single injection of trehalose-6-P into the TPP solution, implying that the purified TPP proteins have detectable phosphatase activity. The ITC titration yielded negative heat deflection, indicating that the intermolecular interaction was an exothermic process. Binding parameters, including $K_d$, binding stoichiometry ($N$), binding enthalpy ($\Delta H$), and binding entropy ($\Delta S$) were obtained by fitting the ITC titration curve using the MicroCal Origin software (Table 2). Our results indicated that trehalose-6-P bound to TPP in the presence of Mg$^{2+}$ with a medium affinity ($K_d = 39.52 \pm 1.78$ $\mu$M) at a molar ratio of 1 : 1.

### Table 2 Binding parameters derived from the ITC titration curve of TPP (100 $\mu$M) with trehalose-6-P (2 mM) in the presence of 2 mM Mg$^{2+}$

<table>
<thead>
<tr>
<th>$N$</th>
<th>$K_d$ (mM)</th>
<th>$\Delta H$ (cal/mol)</th>
<th>$\Delta S$ (cal/mol/deg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.993 $\pm$ 0.017</td>
<td>39.52 $\pm$ 1.78</td>
<td>$-6097$ $\pm$ 1520</td>
<td>$-0.308$</td>
</tr>
</tbody>
</table>

Figure 4 Interactions between TPP and trehalose-6-P Interactions in the absence (A) and presence (B) of Mg$^{2+}$ were detected by ITC at 25°C.

Structure model and the predicted ligand-binding site

I-TASSER is an online unified platform for automatically predicting structures and functions of proteins [11]. We submitted the amino acid sequence of *M. tuberculosis* TPP (otsB2/Rv3372) into the website for predicting 3D structure of TPP. Based on the top 10 threading templates from the RCSB Protein Data Bank such as 1U02, 1UQT, 3T5T, 1GZ5, and so on, 5 structural models of TPP were predicted. Among them, *Thermoplasma acidophilium* TPP (PDB ID 1U02) [19] shared the highest sequence similarity (43%) with *M. tuberculosis* TPP (Fig. 5). In I-TASSER, C-score is used to estimate the quality of the predicted structure model, which is typically in the range of −5 to 2. A model with a higher confidence is signified by a higher value of C-score. Furthermore, the online software is used to provide information about the ligand-binding site of TPP based on similar binding sites of the templates.

The structure with the highest C-score was selected as the best modeling structure of TPP [Fig. 6(A)], with the parameters C-score (−2.96), template modeling score (0.38 ± 0.13), and root-mean-square deviation (14.0 ± 3.9 Å). The best structural model of *M. tuberculosis* TPP contains an Mg$^{2+}$ in the binding pocket. The Mg$^{2+}$ is coordinated by the carboxylate groups of Asp147 and Asp330 and the carbonyl oxygen of Asp149. These three residues Asp147, Asp149, and Asp330 are the conserved residues, corresponding to Asp7, Asp9, and Asp179 in 1U02 [19].

Similar to 1U02, the structural model of *M. tuberculosis* TPP also contains a hydrolase domain and a cap domain. There is a modified Rossmann fold with two extra $\beta$-sheets forming a $\beta$ hairpin in the hydrolase domain. Moreover, the structural model has an extra $\alpha$-helix before the Rossmann fold. However, distinguished from 1U02, the structural
The model of TPP does not contain α/β-fold, which is replaced by three α-helices. Six α-helices, four on one side and two on the other side, flank the five-stranded β-sheet. The cap domain of structural model of TPP is almost the same to that of 1U02, consisting of a four-stranded β-sheet, covered by two α-helices on one side with αβαβ topology. In addition, the structural model of TPP has an extra N-terminal domain composed of a three-stranded β-sheet covered by two α-helices, and three α-helices linking the hydrolase domain. The N-terminal topology is absent in 1U02.

Based on the structural model of TPP, flexible docking was conducted to construct the complex model of TPP binding with trehalose-6-P by using the Simulations/Dock module of the MOE software. The binding pocket of TPP for both trehalose-6-P and Mg²⁺ was determined, that was located on the interface between the hydrolase and the cap domains.

In the structural model of the TPP–trehalose-6-P complex [Fig. 6(B–D)], trehalose-6-P is fitted well in the binding pocket. The sugar group is located at the opening of the cleft between the hydrolase and the cap domains. The phosphate group is buried inside the binding pocket of TPP, forming a hydrogen bond with the residue Gly186. Moreover, four hydrogen bonds are formed between trehalose-6-P and TPP: two from the hydrolase domain (Arg291, Glu295), the other two from the cap domain (Asp149, Arg187).

**Discussion**

In *M. tuberculosis*, OtsAB is the dominant pathway for trehalose biosynthesis, where the TPP protein encoded by the *otsB2* gene represents an attractive anti-tuberculosis drug target [7]. Exploration of the biochemical, ligand-binding, and conformational properties of TPP are crucial for development of a novel drug against *M. tuberculosis*.

In this study, we have successfully expressed and purified the fusion protein of TPP from *M. tuberculosis* (*otsB2/Rv3372*) with pET28a. Using this system, we produced TPP proteins with high purity. MALDI-TOF-MS analysis was also indicative of the high purity of the TPP protein. The S200 gel filtration experiment showed that most of the TPP proteins existed in monomeric state. However, a small part of multimeric TPP proteins were still observed, implying that TPP had a weak tendency to form multimers in solution.

To evaluate the folding of the bacterially produced enzyme, we analyzed the secondary structure of the TPP protein using CD spectroscopy. Our results demonstrated that the recombinant TPP protein adopts α-helix-rich structure, which is consistent with the modeling structure. The structural model shows that TPP is composed of a hydrolase domain, a cap domain, and an N-terminal domain. Similar to template 1U02, the hydrolase and cap domains compose the functional region of TPP, forming a cavity for binding substrate. In addition, TPP contains an extra N-terminal domain, which is not involved in binding either Mg²⁺ or trehalose-6-P. The structural model illustrates that the N-terminal domain is somewhat far away from the other two domains, which might potentially possess other unknown functions or be related to structural integrity.

On the other hand, both the CD and ITC titration experiments demonstrated that Mg²⁺ might be crucial for the structural integrity and catalytic activity of TPP. The Mg²⁺ is...
Located in the binding pocket of the structural model of TPP. Generally, a more open substrate-binding cavity could accommodate more different, larger sugar phosphates. In the structural model of the TPP–trehalose-6-P complex, the substrate-binding cavity of TPP appears to fit trehalose-6-P tightly, suggesting that this protein might be somewhat substrate-specific for binding ligands. This conjecture is also supported by the previous work about TPP, which demonstrated that TPP had high binding specificity for trehalose-6-P as the substrate [8]. Our results demonstrated that TPP possesses a medium affinity for trehalose-6-P in the presence of Mg$^{2+}$.

In summary, by a combination of experimental and computational approaches, we have addressed the conformational and biochemical characteristics of TPP. Our results provide insights into the structural and functional properties of TPP, and may be beneficial for TPP-related novel drug development.

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References


